0273-2289/04/114/0469–0483/$25.00

Thermal Stability of Recombinant Green Fluorescent Protein (GFPuv) at Various pH Values

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Abstract

The thermal stability of the recombinant green fluorescent protein (GFPuv) expressed by *Escherichia coli* cells and isolated by three-phase partitioning extraction with hydrophobic interaction chromatography was studied. The GFPuv (3.5–9.0 μg of GFPuv/mL) was exposed to various pH conditions (4.91–9.03) and temperatures (75–95°C) in the 10 m*M* buffers: acetate (pH 5.0–7.0), phosphate (pH 5.5–8.0), and Tris-HCl (pH 7.0–9.0). The extent of protein denaturation (loss of fluorescence intensity) was expressed in decimal reduction time (*D*-value), the time exposure required to reduce 90% of the initial fluorescence intensity of GFPuv. For pH 7.0 to 8.0, the thermostability of GFPuv was slightly greater in phosphate buffer than in Tris-HCl. At 85°C, the *D*-values (pH 7.1–7.5) ranged from 7.24 (Tris-HCl) to 13.88 min (phosphate). The stability of GFPuv in Tris-HCl (pH > 8.0) was constant at 90 and 95°C, and the *D*-values were 7.93 (pH 8.38–8.92) and 6.0 min (pH 8.05–8.97), respectively. The thermostability of GFPuv provides the basis for its potential utility as a fluorescent biologic indicator to assay the efficacy of moist-heat treatments at temperatures lower than 100°C.

Index Entries: Green fluorescent protein; thermal stability; decimal reduction time; three-phase partitioning; fluorescence intensity; acetate; phosphate.

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Introduction

The biotechnological application of proteins to monitor the preservation of manufactured and processed products has expanded rapidly. Moist-heat treatments using steam at low temperatures ($T \leq 100°C$; pasteurization, blanching, and disinfection) have been applied to food and pharmaceuticals to hinder microbial growth and inactivate endogenous enzymes to preserve and expand product shelf life. Moist-heat disinfection is also utilized for the preservation of low-acid products (pH > 4.5) such as milk and other dairy products. Blanching efficiency is evaluated through the enzyme activity of the most thermostable enzyme present in the product and this enzyme is usually selected and employed as the biologic indicator (BI) to evaluate the immediate efficacy of thermal procedures, as well as to ensure the satisfactory preservation and shelf life of the product. These endogenous thermostable enzymes have been observed to exhibit greater thermal resistance than intact bacteria and yeast cells present in the product.

The high thermostability of lysozyme, an antimicrobial enzyme with considerable potential as a natural food preservative in vegetables, fresh fruits, seafood, and wine, makes it attractive for use in pasteurization processes, reducing nutritional and esthetic quality loss *(1)*. Alkaline phosphatase, lactoperoxidase, and other enzymes have been used for many years as an index of adequate pasteurization of milk *(2)*. Pasteurization associated with the blanching processing of fruit juices is necessary to prevent microbiologic spoilage and to inactivate endogenous enzymes *(3)*. The pectinesterases are an example of endogenous enzymes that also have an important influence on the quality and stability of processed products *(4)*. The dependence of the conditions of the protein inactivation thermal process (time and temperature) varies with pH *(5)* and the heating medium.

Because of the importance of validating the moist-heat treatments and related processes, it is necessary to develop a BI that can efficiently assess the success of the treatment. The use of a fluorescent marker designed for a quick and reliable assay, detectable by microscopy, spectrofluorometry, or a handheld UV lamp, is examined herein. GFPuv provides the basis for its potential utility as a fluorescent BI, to monitor moist-heat treatments ($T < 100°C$). GFPuv is a compact, globular acidic protein (p*I* 4.6–5.4) with one fluorophore consisting of a cyclic tripeptide in the primary protein sequence, a chain of 238 amino acids. It has been shown to be resistant to heat ($T > 70°C$) and alkaline pH (between 5.5 and 12.0; optimum = 8.0).

The term *thermal stability* (also *thermostability*) refers to the resistance of a protein to adverse intrinsic and extrinsic environmental influences, i.e., the thermal characteristic of the protein to remain steady against the denaturation of its molecular integrity and inactivation of its biologic activity on facing high temperatures or other deleterious agents *(6)*. One of the most important indices to measure protein stability is the decimal reduction time, or *D*-value, the time required to reduce 90% of the initial protein concentration exposed to the reference temperature. The *D*-value was used

as the main kinetic parameter to compare the influence of environmental conditions on the thermal behavior of GFPuv.

The purpose of the present work was to determine the thermal stability of the three-phase partitioning (TPP)–extracted recombinant protein, GFPuv, in different buffers, as well as to verify its utility as a quick, accurate, and economical BI for moist-heat treatments ($T < 100°C$) in different pH conditions.

Materials and Methods

Transformation and Expression

Escherichia coli DH5-α cells *(7)* transformed (pGFPuv; Clontech, Palo Alto, CA) by the standard calcium chloride method *(8,9)* to express GFPuv (excitation/emission maxima at 394 /509 nm) were grown (37°C, 100 rpm) in Luria-Bertani broth (USB, Cleveland, OH) supplemented with 100 µg/mL of ampicillin (amp) (Boehringer, Mannheim, Germany) until the broth culture attained $OD_{660} = 0.8$ (10^7 CFU/mL). Isopropyl-β-D-thiogalactopyranoside (USB) was then added to a final concentration of 0.5 m*M*. After 24 h of incubation, brightly green fluorescent cells were harvested by centrifugating at 6.000*g* for 30 min at 4°C. The supernatant was decanted and the cell pellet was resuspended in 4 mL of chilled (4°C) extraction buffer XE (25.0 m*M* Tris-HCl, pH 8.0; 1.0 m*M* β-mercaptoethanol [Pharmacia Biotech, Uppsala, Sweden]; 0.1 m*M* phenylmethylsulfonyl fluoride, [USB]) prior to subjecting the cells to the TPP extraction method.

TPP Extraction Method (10,11) *and Hydrophobic Interaction Chromatography Column Purification*

In a 2-mL microcentrifuge tube, to each 450-µL aliquot of resuspended cells, 300 µL of 4 *M* $(NH_4)_2SO_4$ and 750 µL of *t*-butanol were added. The mixture was stirred for 2 min at 25°C, centrifuged, and then the three phases formed were collected separately. The upper phase of *t*-butanol and the white interfacial precipitate were removed and discarded. A second aliquot of 750 µL of *t*-butanol was mixed into the lower aqueous phase. The mixture was allowed to settle to visible phase separation and centrifuged. At the separation of the three phases, the upper phase was discarded. The interfacial green phase was collected and dissolved in 500 µL of XE. While the lower phase was still fluorescent, it was subjected to repeated TPP extraction. A 1.0-mL aliquot of protein extract was mixed with 1.0 mL of 4 *M* $(NH_4)_2SO_4$, and this mixture was transferred to the top of a methyl support hydrophobic interaction chromatography (HIC) column, fast flow, for final purification. The HIC column was previously equilibrated with 2 *M* $(NH_4)_2SO_4$. The loaded column was first washed with 250 µL of 1.3 *M* $(NH_4)_2SO_4$ to elute proteins that bind with low affinity to the methylated resin of the column packing. GFPuv was eluted with 1.0 mL of buffer solution (10 m*M* Tris-HCl; 10 m*M* EDTA, pH 8.0) and stored at 4°C.

Measurement of Fluorescence Intensity

The fluorescence intensity (±0.005) of GFPuv was measured in the eluted samples in a spectrofluorometer (excitation/emission maxima, 394/509-nm filters, RF 5301 PC; Shimadzu, Kyoto, Japan). Purified recombinant GFPuv (standard GFPuv; Clontech) was used to generate a standard curve to determine TPP-extracted GFPuv concentration and provide an experimental comparison for thermal stability. The fluorescence intensity (*I*) of the TPP-extracted samples was compared with the standard calibration curve Eq. 1:

$$I = -33.078 + 38.943 \times (\text{GFPuv } \mu\text{g/mL})$$
$$R^2 = 0.997 \quad (1)$$

To determine the concentration of the TPP-extracted GFPuv, fluorescence intensity was correlated to the concentration of native GFPuv, since denatured GFPuv cannot fluoresce, and the loss in fluorescence intensity was a measure of denatured GFPuv concentration.

Moist-Heat Treatment of Extracted GFPuv Under Different pH Conditions

Samples of purified GFPuv were exposed (24 h, 4°C) to different pH conditions (5.0 ± 0.2 to 9.0 ± 0.2) in 10 m*M* buffer solutions before heating: (1) acetate (pH 5.0–7.0), (2) phosphate (pH 5.5–8.0), and (3) Tris-HCl (pH 7.0–9.0). To each 3900 µL of buffer solution, 100 µL of TPP-extracted sample (at an initial concentration of about 420 µg of GFPuv/mL) was added. The mixture (4000 µL) was stored at 4°C overnight and centrifuged (6000*g* for 30 min). Precipitation was not observed. The pH values of the buffer solutions were also measured and correlated with the correspondent µg of GFPuv/mL by the standard calibration curve (Eq. 1). A pHmeter AR-20 (Fisher) was previously adjusted with standard buffers (Synth, Saõ Paulo, Brazil) of known pH values: 4.0, 7.0 and 9.0. One-milliliter samples were transferred to quartz cuvets ([3 × 10 mm] light path length × 45 mm height) and sealed with plastic covers. Each cuvet was inserted into the adapter assembly and adjusted in the cell holder. The cell holder was connected to a constant-temperature bath and the circulating water stabilized at the assay temperature. A constant-temperature water-circulating device (Thermo-bath TB-85 P/N 200-65022; Shimadzu), was used to heat and control the temperature of the cell holder. The temperature of the water bath was maintained within ±0.05°C and provided constant temperature by continuous circulation of water from the water bath to the cell holder and the sample in the cuvet via a circulation pump. From the moment the sample-filled cuvets were placed in the cell holder and the heat treatment was initiated, a fluorescence reading was taken at defined exposure intervals at different temperatures (75, 80, 85, 90, and 95°C). The variation in fluorescence intensity (±0.005) was measured by

a spectro-fluorometer every 20 s and converted to native GFPuv concentrations by the standard calibration curve (Eq. 1).

Analysis of Thermal Kinetic Parameters

The GFPuv inactivation curves were considered first-order models represented by

$$\mathrm{Log}_{10} C_f = \mathrm{Log}_{10} C_0 - (1/D) \times t = \mathrm{Log}_{10} C_0 - (k/2.303) \times t \quad (2)$$

in which C_0 is the initial concentration of native GFPuv (from 3.5 to 9.0 µg of GFPuv/mL); and C_f is the final concentration of GFPuv (from 1.00 to 1.55 µg of GFPuv/mL), after exposure to heating time (t, min), D-value (h or min), and inactivation rate constant (k, h^{-1}, min^{-1}). The D-value, the interval of time required to reduce one decimal logarithm of the initial fluorescence intensity of GFPuv at reference temperature, was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the inactivation curves (Log_{10} µg of GFPuv/mL vs time of exposure at a constant temperature). The temperature coefficient, Q_{10}, was calculated to evaluate the dependence of the D-value on an increase of 10°C in the heating temperature (T, °C), by the following relation:

$$Q_{10} = \left| D_T / D_{(T+10)} \right| = \left(10^{10/z} \right) \quad (3)$$

The z-value, expressed as the number of degrees (°C) required for one Log_{10} change in the D-value (D = 2.303/k), was determined by plotting the relation between Log_{10} D-value and corresponding temperatures. The z-value may be related to the coefficient Q_{10} of the process by $Q_{10} = (10^{10/z})$. Activation energy (E_a, kcal/mol) was estimated by using the Arrhenius equation, which was applied to evaluate the dependence of the inactivation rate constant, k, on the heating temperature:

$$\mathrm{Log}_{10} k_1 = \mathrm{Log}_{10} k_2 - \left\{ \left(E_a / 2.303 \times R \right) \left[\left(1/T_1 \right) - \left(1/T_2 \right) \right] \right\} \quad (4)$$

in which T_1 and T_2 are the heating temperatures in degrees Kelvin (K), and R is the universal gas constant (1.987 calories × mol^{-1} K^{-1}).

Results

Stability in Buffers

GFPuv, expressed by *E. coli*, isolated by the TPP-extraction method and eluted from a methyl HIC column (extracted GFPuv), was diluted in buffers and stored at 4°C to stabilize the solution pH values before heating. After 24 h at 4°C, the concentration of GFPuv varied in accordance with buffer solution and respective pH values. In acetate buffer, the lower GFPuv concentration ranged from 1.34 to 5.87 µg of GFPuv/mL at pH between 4.91 and 5.72, and the higher GFPuv concentration ranged from 6.20 to 9.20 µg of GFPuv/mL at pH between 6.13 and 7.03. In phosphate buffer, the lower GFPuv concentration ranged from 4.89 to 5.96 µg of GFPuv/mL at pH

between 5.67 and 5.97, and the higher GFPuv concentration ranged from 5.31 to 9.08 μg of GFPuv/mL at pH between 6.19 and 8.00. In Tris-HCl buffer, the GFPuv concentration ranged from 5.52 to 9.33 μg of GFPuv/mL at pH between 6.67 and 9.03. The greater loss of fluorescence intensity for GFPuv in acetate buffer was about 74% at pH 4.92, lowering the loss to 49% at pH 5.32, decreasing to 10% of loss at pH 5.66 and to 2.0% at pH > 6.0. The stability of fluorescence intensity was verified for GFPuv in phosphate and Tris-HCl buffers at pH ≥ 6.40, and the maximum losses of fluorescence intensity in both buffers occurred at about 15.0 ± 5.0%. The effect of pH on the standard and TPP-extracted/purified GFPuv was previously studied and confirmed that a quite good, stable fluorescence intensity for GFPuv was characterized in the pH range of 6.0–9.8, declining abruptly between pH 5.5 and 4.5, with a minimum fluorescence intensity at pH < 4.5 *(9)*. Bokman and Ward *(12)* verified that native GFPuv maintains stable fluorescence in the pH range of 5.5–12.0; however, fluorescence intensity decreases between pH 5.5 and 4.4 and drops sharply above 12.0.

Thermal Treatments

The variation in fluorescence intensity was measured by a spectrofluorometer and converted to the concentration of native GFPuv. After heating and repeated exposure to light during the assay, the GFPuv samples were stored for 24 h at 4°C, and fluorescence intensity was again measured and compared to final heat-treatment intensity. No increase in fluorescence inten-sity was observed that may be related to the recovery of the native conformation of the protein. Postheating, the pH did not change and the samples remained clear, without any protein precipitating out of the solution.

D-*Value*

The denaturation of GFPuv in buffer solutions at various pH values was measured by the loss of fluorescence intensity and expressed in the decimal logarithm of the decrease in native GFPuv concentration vs the time of exposure at constant temperature (Fig. 1). To estimate *D*-values at constant heating temperatures and pH values (Table 1), the interval of GFPuv concentrations considered was between 3.5 (acetate, pH 5.18) and 9.0 μg/mL (Tris, pH > 8.0, for initial concentration [C_o]) and ranged from 1.00 to 1.55 μg/mL for final concentration (C_f), which corresponded to the linear portion of the inactivation curves (Log_{10} μg of GFPuv/mL vs time of exposure at a constant temperature and pH value; Fig. 1).

Fig. 1. *(opposite page)* Inactivation curves for GFPuv in **(A)** acetate buffer at pH 6.0, **(B)** phosphate buffer at 85°C and different pH values, **(C)**Tris-HCl at pH 8.5. The *D*-values were determined from the reciprocal of the slope of the linear portion of the GFPuv inactivation curves, considered first-order models.

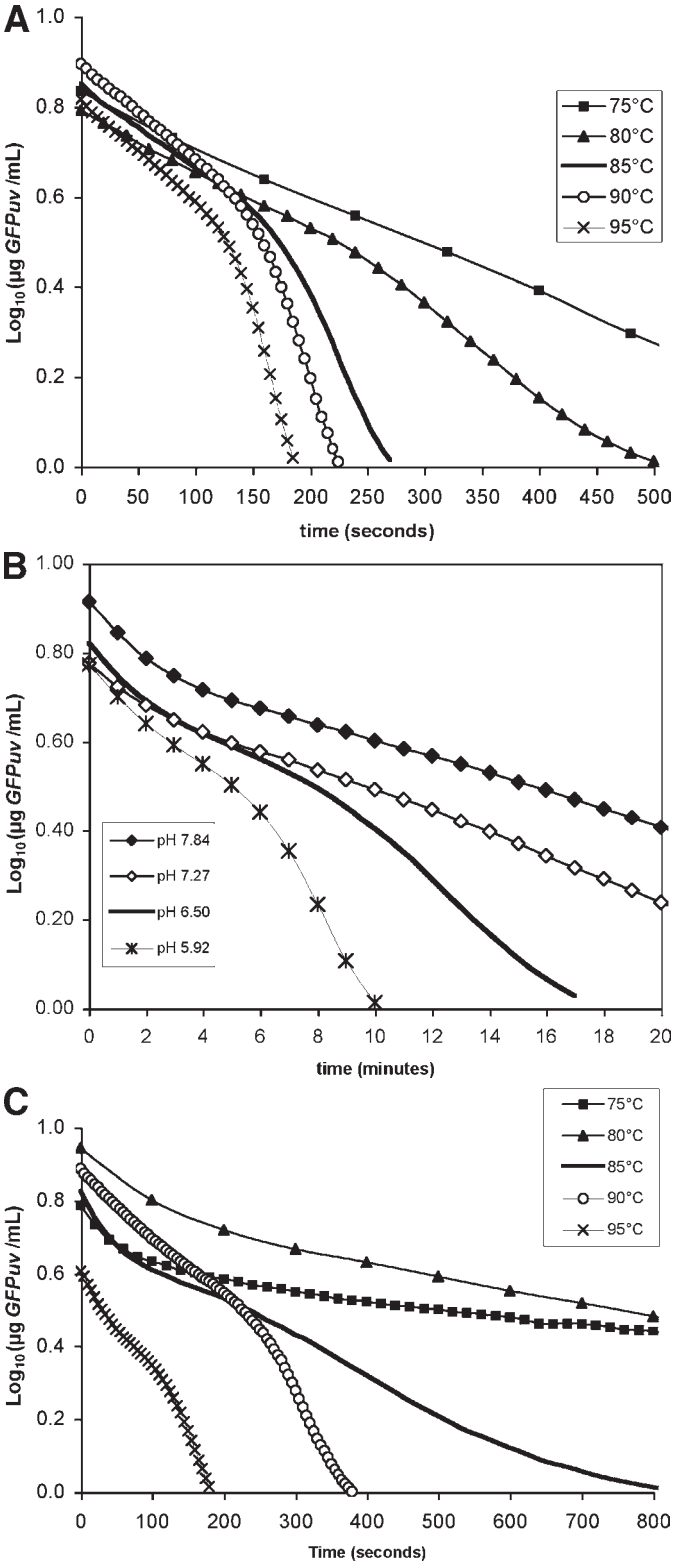
A
1.0
0.8
0.6
0.4
0.2
0.0
Log10 (µg GFPuv /mL)
0 50 100 150 200 250 300 350 400 450 500
time (seconds)
75°C
80°C
85°C
90°C
95°C
B
1.00
0.80
0.60
0.40
0.20
0.00
Log10 (µg GFPuv /mL)
0 2 4 6 8 10 12 14 16 18 20
time (minutes)
pH 7.84
pH 7.27
pH 6.50
pH 5.92
C
1.0
0.8
0.6
0.4
0.2
0.0
Log10 (µg GFPuv /mL)
0 100 200 300 400 500 600 700 800
Time (seconds)
75°C
80°C
85°C
90°C
95°C

Table 1
D-values at Reference Temperatures and pH Intervals[a]

Temperature (°C)	Buffer solution (10 m*M*)	*D*-value (min)[b]							
		5.0–5.5	5.6–6.0	6.1–6.5	6.6–7.0	7.1–7.5	7.6–8.0	8.1–8.5	8.6–9.0
75	Acetate	7.60 ± 2.39	7.93 ± 2.40	21.40 ± 7.13	41.60 ± 0.48				
	Phosphate		12.89 ± 1.12	11.54 ± 3.94	58.33 ± 28.29	76.38 ± 13.61	104.16 ± 40.83		
	Tris-HCl				25.7 ± 22.04	30.52 ± 3.19	69.44 ± 15.72	125.00 ± 47.15	52.08 ± 35.36
80	Acetate	5.74 ± 1.12	7.24 ± 1.20	10.14 ± 1.50	17.95 ± 3.23	20.80 ± 2.20			
	Phosphate			9.72 ± 0.55	23.80 ± 0.62	55.55 ± 0.73	84.15 ± 0.96		
	Tris-HCl					23.83 ± 0.75		55.55 ± 0.64	55.55 ± 0.42
85	Acetate	3.71 ± 0.80	4.76 ± 0.15	5.55 ± 0.23	7.09 ± 0.17	8.33 ± 0.10			
	Phosphate		4.38 ± 0.36	6.40 ± 0.96	13.88 ± 0.15	13.88 ± 0.05	17.67 ± 1.65		
	Tris-HCl					7.24 ± 1.20	10.57 ± 1.50	18.51 ± 1.10	27.75 ± 0.06
90	Acetate	3.26 ± 0.80	3.96 ± 0.45	4.62 ± 0.10	5.13 ± 0.08	5.05 ± 0.13			
	Phosphate			4.38 ± 0.09	5.74 ± 0.07	6.66 ± 0.05	6.94 ± 0.09		
	Tris-HCl					3.56 ± 0.73	4.91 ± 0.16	6.25 ± 1.29	5.5 ± 2.62
95	Acetate	3.74 ± 0.58	4.06 ± 0.47	4.38 ± 0.32	4.70 ± 0.23	4.9 ± 0.26			
	Phosphate		3.78 ± 0.05	4.44 ± 0.07	5.96 ± 0.24	5.95 ± 0.33	6.08 ± 0.38	5.42 ± 0.06	
	Tris-HCl			2.92 ± 0.72		2.46 ± 0.69	3.35 ± 0.79	5.17 ± 2.51	5.48 ± 2.05

[a] $n = 10$ observations; $p < 0.05$.
[b] ($D \pm$ CI) min.

At 90°C and 95°C, a slight drop in fluorescence intensity was observed (Fig. 1) without interfering with the determination of *D*-value. For lower temperatures, the loss of fluorescence intensity decreased gradually. For the same temperatures, the thermal stability of GFPuv was observed to gradually increase proportionally with pH and was shown to be dependent on the buffer used.

At 75°C, the mean *D*-values for GFPuv attained their maximum in acetate at pH 6.6–7.0 (D = 41.60 min), doubled in phosphate at pH 7.6–8.0 (D = 104.16 min), and increased threefold in Tris-HCl at pH 8.1–8.5 (D = 125.00 min). The phosphate buffer system was shown to provide greater thermal stability to the protein between pH 6.6 and 8.0. However, at pH 6.1–6.5, the *D*-values for GFPuv in acetate were double those for GFPuv in phosphate, and at 6.6–7.0 the thermal behavior of GFPuv was equivalent for both buffers.

At 80 and 85°C, respectively, the *D*-values for GFPuv in acetate (D = 20.80 and 8.33 min) and in Tris-HCl (D = 23.83 and 7.24 min) at pH 7.1–7.5 were very close and half the *D*-values (D = 55.55 and 13.88 min) observed in phosphate. Therefore, the phosphate buffer provided the optimal buffering system to maintain the thermal GFPuv stability in an important range of pH 6.0–8.0. At ≥85°C, *D*-values dropped at least twice for every buffer system. The *D*-values were similar for GFPuv in phosphate and acetate buffers at 90–95°C, pH 5.5–7.0. The thermal stability of GFPuv was the highest in the phosphate buffer at pH 7.6–8.0 and Tris-HCl at pH > 8.0. At pH 7.6–8.0 in phosphate and pH > 8.0 in Tris-HCl buffer, the highest thermal stability of GFPuv was shown to be equivalently maintained.

Correlation of D*-Value with pH and Buffer Systems*

A good stability of the GFPuv molecule was observed *(9)* to start at pH about 5.0–5.2. This corresponds to our findings on the minimum thermal GFPuv stability that was shown at pH 4.91, referent to acetate buffer and treatment at 85°C, very close to the p*I* of the protein, which was verified *(13)* between pH 4.7 and 5.1, corresponding to our finding on GFPuv extraction efficiency.

The fluorescence intensity of GFPuv demonstrated thermal stability at pH > 6.0. However, at pH 8.5–9.0, the fluorescence intensity was variable at 75°C in Tris-HCl and was kept constant for the higher temperatures, considering the same buffer system. Therefore, the range of pH for different buffers and treatments was linear correlated to *D*-values, which proportionally to the increase in pH followed higher thermal GFPuv stability.

The linear correlation between extreme pH intervals and *D*-values was shown to be dependent on buffer systems and heating temperatures, as shown in Table 2, from (1) pH 4.91 (85°C) to 7.03 (85°C) for acetate, (2) pH 5.63 (85°C) to 7.91 (80°C) for phosphate, and (3) pH 6.91 (75°C) to 9.03 (90°C) for Tris-HCl solutions.

Table 2
Equations to Determine *D*-values for GFPuv in Various Buffers Exposed to Different Conditions of Temperature and pH

Buffer solution	Temperature (°C)	pH range	*D*-value equation	R^2
Acetate	75	5.5–7.01	–138.09 + 25.677 × pH	0.97
	80	5.18–7.01	–41.644 + 8.8106 × pH	0.94
	85	4.91–7.03	–12.1 + 2.8747 × pH	0.94
	90	5.19–7.01	–1.9854 + 1.0318 × pH	0.97
	95	5.46–7.01	–0.9143 + 0.8296 × pH	0.91
Phosphate	75	6.19–7.20	–392.14 + 64.603 × pH	0.92
	80	5.97–7.91	–271.76 + 45.632 × pH	0.96
	85	5.63–7.84	–35.676 + 6.8552 × pH	0.94
	90	6.03–7.80	–5.6922 + 1.6322 × pH	0.93
	95	5.89–7.82	–3.6824 + 1.3041 × pH	0.97
Tris-HCl	75	6.91 – 8.50	–636.99 + 92.299 × pH	0.89
	80	7.50–8.84	–182.68 + 27.510 × pH	0.91
	85	7.41–8.90	–91.089 + 13.097 × pH	0.94
	90	7.27–9.03	–14.998 + 2.5629 × pH	0.77
	95	7.08–8.92	–30.48 + 4.5551 × pH	0.98

Kinetic Parameters Z-Value, Q_{10} Coefficients, and E_a

The kinetic parameters *z*-value and the related Q_{10} coefficient ($Q_{10} = 10^{10/z}$) reflect the temperature dependence of the denaturation phenomenon (decrease in fluorescence intensity). The *z*-value is the interval of temperature required to change the *D*-value by a factor of 10.

For every temperature and buffer, the corresponding *D*-values at pH between 6.0 and 8.5 were calculated from the set equations shown in Table 2. The respective *z*-values (°C), were determined from the negative reciprocal of the slopes of the regression lines of the relations between Log_{10} *D*-value and corresponding temperatures. The Q_{10} coefficients were estimated through the relation to *z*-values ($Q_{10} = 10^{(10/z)}$); those parameters are given in Table 3.

The thermal stability of GFPuv was closely maintained at pH > 6.0 in the buffers: (1) acetate at pH 6.5 (z = 23.42°C) and pH 7.0 (z = 20.45°C); (2) phosphate at pH 7.0 (z = 16.64°C), pH 7.5 (z = 14.01°C), and pH 8.0 (z = 13.75°C); (3) Tris-HCl at pH 7.5 (z = 15.41°C), pH 8.0 (z = 15.60°C), and pH 8.5 (z = 14.95°C). At pH 6.0, the *z*-value of 30.96°C for GFPuv in acetate led to the highest thermal stability of the protein for the temperature interval between 75 and 95°C. The *z*-values dropped about 25% at pH 7.0 and 7.5, from z = 23.42°C (acetate) and 22.42°C (phosphate) to z = 20.45°C (acetate) and 20.79°C (Tris-HCl), respectively. At pH 8.0, the *z*-values dropped by half, between 13.75 and 15.60°C considering phosphate and Tris-HCl buffers, respectively.

Table 3
z-value, Q_{10} Coefficients, and E_a of Thermal Stability for GFPuv

	Acetate			Phosphate			Tris-HCl		
pH	*z*-value (°C)	Q_{10}	E_a (kcal/mol)	*z*-value (°C)	Q_{10}	E_a (kcal/mol)	*z*-value (°C)	Q_{10}	E_a (kcal/mol)
6.0	30.96	2.10	19.27						
6.5	23.42	2.67	25.15	22.42	2.79	26.18			
7.0	20.45	3.08	28.76	16.64	3.99	35.28	20.79	3.03	28.19
7.5				14.01	5.17	42.63	15.41	4.46	38.15
8.0				13.75	5.34	42.67	15.6	4.38	38.2
8.5							14.95	4.67	39.9

Meanwhile, the process coefficient Q_{10} ($Q_{10} = 10^{10/z}$) of about 3.0 suggested that acetate and Tris-HCl buffers at pH 7.0 provided similar stabilization for the protein's thermal characteristics greater than phosphate ($Q_{10} = 3.99$), whose system exposed GFPuv to more dependence on temperature change. At pH 8.0, the protein in Tris-HCl was confirmed to exhibit greater stability than in phosphate, when the *D*-values decreased, respectively, four and five times for every 10°C increase in heating temperature.

The rate of inactivation dependent on treatment temperatures can also be described by either activation energy (E_a) or the Arrhenius equation. E_a, which relates the intrinsic energy of the system, indicates the stability of the system during heat treatment. Therefore, stable systems possess lower energy than unstable ones. E_a and *z*-value are related: inactivation reactions that exhibit large *z*-value and small E_a are less influenced by temperature; however, systems that have small *z*-value and high E_a have greater temperature susceptibility (Table 3).

The stability of GFPuv was greater in acetate at pH 6.0 ($E_a = 19.27$ kcal/mol), and equivalent ($E_a \sim 26.50$ kcal/mol) at pH 6.5 in acetate and phosphate and at pH 7.0 in acetate and Tris-HCl. For the same buffers, on the increase in pH to 7.5–8.5, the thermal vulnerability of GFPuv incremented 30% in the E_a range from 38.15 to 39.9 kcal/mol. Otherwise, the solutions of GFPuv in phosphate at pH 7.5 and 8.0 (E_a = 42.63 and 42.67 kcal/mol, respectively) established the lowest thermal stability for GFPuv.

For pH > 7.0, the $Q_{10} > 5.0$ coefficient showed *D*-values 13% more vulnerable for GFPuv in phosphate than in Tris-HCl and acetate buffers, confirming its role in the improved maintenance of GFPuv conformation and fluorophore emission output.

The thermal stability of GFPuv was similar for the following systems: (1) acetate solutions at pH interval of 6.5–7.0 and respective *z*-values of 23.42 and 20.45°C; (2) phosphate solutions between pH of 7.0 and 8.0 and respective *z*-values of 16.64, 14.01, and 13.75°C; (3) Tris-HCl at pH interval of 7.5–8.5 and respective *z*-values of 15.41, 15.60, and 14.95°C.

The thermal stability of GFPuv was (1) higher when heated in acetate solution pH 6.0 (E_a = 19.27 kcal/mol), and (2) equivalent for the systems provided by acetate solutions at the pH interval of 6.5–7.0 (E_a = 25.15 and 28.76 kcal/mol), phosphate at pH 6.5 (E_a = 26.18 kcal/mol), and Tris-HCl at pH 7.0 (E_a = 28.19 kcal/mol).

The data confirmed the previous *(14)* thermal stability of standard GFPuv in Tris-HCl buffer at pH 8.5, when the *z*-value was about 12.9°C (Q_{10}= 6.0, E_a = 45.54 kcal/mol), comparable with an average of z = 13.75°C (Q_{10}= 5.34) observed for the extracted protein heated in phosphate buffer at pH 8.0, and the system corresponded to an average E_a = 42.67 kcal/mol, for the same interval of temperature, between 75 and 95°C.

Discussion

Laratta et al. *(15)* studied five tomato varieties and all varieties showed the same thermal performance: the thermal pectin methyl esterase resis-

tance was higher at higher pH. All pectin methyl esterase varieties showed a typical biphasic performance, characterized by a lower thermal resistance at temperatures lower than 74°C and a higher thermal resistance above that temperature. The temperature range between 90 and 115°C is generally employed in the food industry to microbiologically and enzymatically stabilize these products. The *z*-values for the five varieties of tomatoes varied from 18.9 to 36.0°C related to a pH range of 3.9–4.9, which was responsible, in our point of view, for a low interval of *D*-values, between 0.16 and 0.18 min. The variation in thermal stability of GFPuv verified in the present work related to the three buffers was similar considering the *z*-value range from 13.75°C in phosphate (pH 8.0) to 30.96°C for acetate (pH 6.0) solutions, corresponding to an interval of *D*-value between 4.06 (pH 5.6–6.0) and 6.08 min (pH 7.6–8.0) at 95°C.

Forsyth et al. *(16)* studied the thermal performance of peroxidase at 75°C and obtained a *D*-value range from 0.01 to 7.38 min for the native enzyme and between 0.096 and 18.96 min for the partially inactivated enzyme.

Griffiths *(2)* studied the thermal stability of the naturally occurring enzymes in milk at temperatures between 65 and 80°C, in order to choose an enzymatic index of adequate pasteurization of milk. For the acid phosphatase, the *D*-values of 7.38 min at 75°C and 7.87 min at 80°C corresponded to a *z*-value of 6.6°C; for the inactivation of lactoperoxidase, *D*-values of 0.80 min at 75°C and 0.075 min at 80°C corresponded to a *z*-value of 5.4°C; for amylase (saccharifying activity), *D*-values of 0.85 min at 75°C and 0.45 min at 80°C corresponded to a *z*-value of 16.2°C. The naturally occurring enzymes in milk showed lower *D*-values than those found for GFPuv in the three buffers. However, a *z*-value of 16.64°C characterized for GFPuv in phosphate buffer solutions at pH 7.0 was similar to that obtained for amylase in milk.

The thermal kinetics for lysozyme in beer *(1)* was characterized for systems containing 15% glucose at pH 5.20 and 7.20, corresponding respectively to *z*-values of 13.8 and 14.1°C, and to E_a values of 42.0 and 41.1 kcal/mol. For systems containing 1.0 *M* NaCl at pH 7.20, the obtained parameters were a *z*-value of 13.9°C and a E_a value of 41.8 kcal/mol. For both systems, the kinetic parameters were similar to those verified for GFPuv in phosphate solutions at pH 7.5 and 8.0.

Thermal inactivation of *Bacillus cereus* spores was confirmed to be affected by the solutes used to control water activity (A_w) of the heating medium. For the spores of *B. cereus* ATCC 7004 subjected to a heating interval between 92 and 106°C, Mazas et al. *(17)* determined the following parameters: (1) *z*-value of 8.3°C for spores in water (A_w = 1.0) at pH 7.60, as a medium control; (2) *z*-value of 10.3°C for spores in 5.6 *M* NaCl solution at pH 8.35 and A_w = 0.75. For both extreme systems, the spores of *B. cereus* exhibited lower thermal stability than even the system of GFPuv in phosphate solution (*z*-value minimum of 13.75°C). Among the environmental conditions that affect spore thermoresistance, Vessoni Penna et al. *(18)*

stated that pH value is a major intrinsic factor in microbial destruction, as well as in the recovery medium of the injured spores.

Since the direct estimation of microbial numbers by conventional culturing methods is time-consuming and well affected by environmental conditions *(18)*, a simple test for the most resistant enzyme activity is appropriate on a routine basis. If enzyme activity is detected, it can be assumed that the heat treatment was inadequate *(4,15)*. Appropriately, GFPuv can be employed as a BI in thermal processes (blanching, pasteurization, disinfection) at temperatures ≥75°C for products at pH > 5.5, with the inactivation of GFPuv shown to be directly related to the time of exposure to moist heat and pH.

Conclusion

The thermal stability of GFPuv provides the basis for its potential utility as a fluorescent BI to assay the efficacy of processes at temperatures lower than 100°C at an interval of pH between 5.5 and 8.5 and dissolved in various buffers.

Acknowledgments

We thank our personal assistants for providing technical support for biologist Irene A. Machoshvili. This study was made possible by financial support provided by the Brazilian Committees for Scientific Technology Research (Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior, Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Fundação de Amparo à Pesquisa do Estado de São Paulo).

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